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Risk Factors for Metachronous Gastric Neoplasms in Patients Who Underwent Endoscopic Resection of a Gastric Neoplasm

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Background/Aims: To identify the risk factors for metachronous gastric neoplasms in patients who underwent an endoscopic resection of a gastric neoplasm. **Methods:** We prospectively collected clinicopathologic data and measured the methylation levels of *HAND1*, *THBD*, *APC*, and *MOS* in the gastric mucosa by methylation-specific real-time polymerase chain reaction in patients who underwent endoscopic resection of gastric neoplasms. **Results:** A total of 257 patients with gastric neoplasms (113 low-grade dysplasias, 25 high-grade dysplasias, and 119 early gastric cancers) were enrolled. Metachronous gastric neoplasm developed in 7.4% of patients during a mean follow-up of 52 months. The 5-year cumulative incidence of metachronous gastric neoplasm was 4.8%. Multivariate analysis showed that moderate/severe corpus intestinal metaplasia and family history of gastric cancer were independent risk factors for metachronous gastric neoplasm development; the hazard ratios were 4.12 (95% confidence interval [CI], 1.23 to 13.87; p=0.022) and 3.52 (95% CI, 1.09 to 11.40; p=0.036), respectively. The methylation level of *MOS* was significantly elevated in patients with metachronous gastric neoplasms compared age- and sex-matched patients without metachronous gastric neoplasms (p=0.020). **Conclusions:** In patients who underwent endoscopic resection of gastric neoplasms, moderate/severe corpus intestinal metaplasia and a family history of gastric cancer were independent risk factors for metachronous gastric neoplasm, and *MOS* was significantly hypermethylated in patients with metachronous gastric neoplasms. **(Gut Liver 2016;10:228-236)**

Key Words: Stomach neoplasms; Metastasis; Risk factors; Therapeutics

INTRODUCTION

Metachronous gastric cancer (MGC) develops in a considerable portion of patients who underwent endoscopic resection (ER) of early gastric cancer (EGC).[1,2] Therefore, it is very important to elucidate risk factors for MGC in these patients to establish an appropriate surveillance strategy. Old age, family history of gastric cancer, extensive corpus atrophy, intestinal metaplasia (IM), and persistent *Helicobacter pylori* infection were suggested as risk factors for MGC in previous studies.[3-5] On the other hand, although the optimal treatment strategy has not yet been established, aggressive treatments such as endoscopic mucosal resection or endoscopic submucosal dissection have been more frequently performed for gastric dysplasia. The reason is that gastric dysplasia is a more advanced premalignant lesion than gastric atrophy/IM; additionally it is focal lesion which makes it easy to try preemptive ER in contrast to gastric atrophy/IM. Therefore, it would be practical to manage EGC and gastric dysplasia in conjunction, as "gastric neoplasm," although the interval of surveillance after ER could vary based on whether the lesion is cancer or dysplasia. However, few studies have evaluated risk factors for metachronous gastric neoplasm (MGN) including dysplasia, in the patients who undergo ER of gastric neoplasm.

Gastric cancer develops through the accumulation of genetic and epigenetic alterations. Recently, attention has focused on aberrant DNA methylation as an important mechanism of gastric carcinogenesis. *H. pylori* infection induces chronic

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inflammation, increased secretion of several cytokines and hypermethylation of promoter regions of tumor suppressor genes. Consequently, tumor suppressor genes are accumulatively inactivated, resulting in the development of gastric cancer. This is a well-known the concept of field cancerization.[6,7] That is, by the time gastric cancer becomes visible, the stomach likely harbors areas containing premalignant lesions.[8] Therefore, we could expect that the higher the aberrant DNA methylation related to gastric carcinogenesis in a patient who underwent ER of gastric neoplasm, the higher the risk of MGN due to field cancerization. However, there are few studies on this topic. IM is one of the strongest risk factors for gastric cancer[9] and it is considered as the key link in the process from *H. pylori* infection to gastric cancer through the aberrant DNA methylation. We have recently elucidated *THBD*, *HAND1*, and *APC* as hypermethylated genes related to IM.[10] Genome-wide DNA methylation profiles in noncancerous gastric mucosae have identified *MOS* as a hypermethylated gene in the gastric cancer irrespective of *H. pylori* infection.[11] In subsequent studies, we found that the methylation level of *MOS* correlated with severity of IM.[12,13] We therefore speculated that *THBD*, *HAND1*, *APC*, and *MOS* which are related to severity of IM and show persistent methylation after *H. pylori* eradication could be molecular risk factors for MGN.

The aim of the current study was to identify risk factors for MGN among diverse clinicopathologic factors and above-mentioned hypermethylated genes in the patients who underwent ER of gastric neoplasm.

MATERIALS AND METHODS

1. Patients

Between October 2004 and July 2013, patients diagnosed with gastric neoplasm by endoscopic biopsy who underwent ER by one experienced endoscopist (N.K.) were prospectively enrolled at Seoul National University Bundang Hospital, Seongnam, South Korea. All participants were ethnically Korean. From this subject pool, only patients who had been followed up by regular endoscopy for more than 12 months were enrolled in the study. Patients were excluded from this study based on the following criteria: (1) patients whose final diagnosis was beyond expanded criteria of endoscopic submucosal dissection for EGC[14] on pathologic review of the resected specimen; and (2) patients who had another underlying cancer. This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (IRB number: B-1403-242-302).

2. Determination of *H. pylori* infection status

To determine *H. pylori* infection status, three biopsy-based tests (histology, rapid urease test, and culture) were used. A total of 10 biopsy specimens were taken from the gastric mucosa of each patient. Among these 10 specimens, four were used for histological evaluation of *H. pylori* infection by modified Giemsa staining (one each from the greater and lesser curvature of the antrum and body). Another four specimens from the four gastric mucosa areas mentioned above were used for *H. pylori* culturing. The remaining two specimens from the lesser curvature of the antrum and body were used for the rapid urease test (CLOtest; Delta West, Bentley, Australia). The protocols for these three biopsy-based tests have been described in detail.[15] Current *H. pylori* infection was defined as positive from any of these three tests.

Two other methods were used to identify patients who had a previous *H. pylori* infection. First, an enzyme-linked immunosorbent assay (ELISA) was used to screen for immunoglobulin G (IgG) specific for *H. pylori* in each patient's serum (Genedia *H. pylori* ELISA; Green Cross Medical Science Corp., Eumsung, Korea). Second, each patient was questioned about his history of *H. pylori* eradication. If the patient had a history of *H. pylori* eradication or *H. pylori* serology was positive but no bacteria were found by histology, the rapid urease test, or culturing, the patient was diagnosed with a past *H. pylori* infection without current ongoing infection.[16]

3. Evaluation of gastric atrophy and IM

The severity of gastric atrophy in each patient was evaluated by the serum pepsinogen (PG) test. Fasting serum was collected from the patients, and serum concentrations of PG I and II were measured using a Latex-enhanced Turbidimetic Immunoassay (Shima Laboratories, Tokyo, Japan). Based on the results of the serum PG tests, the patients were categorized as having no, mild to moderate, or severe gastric atrophy according to the definition of Miki *et al*.[17] No atrophy was defined as PG I >70 and PG I/II ratio >3.0. Severe atrophy was defined as PG I ≤30 and PG I/II ≤2.0. All other patients were identified with mild to moderate atrophy.

To evaluate the severity of IM, we recorded the updated Sydney system scores of the four biopsy specimens from each patient used for histological evaluation of *H. pylori* infection.[18]

4. ER and follow-up

Each patient underwent one of two types of ER. Endoscopic mucosal resection was performed usually for the small gastric dysplasia. For dysplasia larger than 2 cm and most EGCs, endoscopic submucosal dissection was preferred. The technical methods of endoscopic mucosal resection and endoscopic submucosal dissection were previously described.[19]

During the follow-up period, endoscopy was performed routinely at 3, 6, and 12 months, and then annually to assess the completeness of resection as well as to detect metachronous lesions. Biopsy samples were taken from the scar of ER or other suspicious mucosal abnormalities. Abdominal computed tomography and chest radiography were performed annually to assess distant metastases. MGN was defined as gastric dysplasia or

cancer which was developed at least 12 months after initial ER of gastric neoplasm.

5. Quantitative methylation-specific polymerase chain reaction

Patients with and without MGN among the included subjects were matched for sex and age by 1:1. In these patients, methylation-specific polymerase chain reaction (PCR) was performed and the methylation level of each gene was compared between the two groups. The protocols for DNA preparation and quantitative methylation-specific PCR have been described in detail.[10] Briefly, genomic DNA was extracted directly from nonneoplastic antral biopsy specimens. After isolation, the DNA was subjected to sodium bisulfite modification.[20] DNA methylation in the four selected CpG sites (*HAND1*, *THBD*, *APC*, and *MOS*) were evaluated as previously.[10,11] The primer sequences for methylation-specific PCR were designed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). An aliquot of 2 μL was used for real-time PCR with a primer set specific to methylated or unmethylated sequences, with a specific annealing temperature of 53°C to 66°C. Real-time PCR was performed using 23SYBRV® Premix Ex Taq™ (Takara Bio, Shiga, Japan) and ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster, CA, USA). Standard DNA was prepared by cloning PCR products into the pGEM-T Easy vector (Promega, Madison, WI, USA). The number of molecules in a test sample was determined by comparing the amplification with those of standard samples containing a known number of molecules (10^6–10^1). The number of methylated and unmethylated molecules was measured separately, and the methylation level was calculated as following: methylation level=number of methylated molecules/total number of DNA molecules (methylated+unmethylated molecules).

6. Statistical analysis

SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Baseline clinicopathologic characteristics of the patients were presented as descriptive data. Cumulative probabilities of MGN were estimated by the Kaplan-Meier method. The log-rank test was used to compare the time-to-event curves of MGN according to the severity of IM in the gastric body. A univariate Cox proportional hazards model was used to identify possible covariates as significant risk factors for MGN. Then, the variables with $p<0.05$ were subjected to multivariate Cox proportional hazards model to identify independent contribution. In addition, the variables which were considered to be possible risk factors for MGN based on the previous studies were also analyzed in multivariate model. The methylation level of four genes in patients with and without MGN were compared by Mann-Whitney U test, as the data from the two groups were not normally distributed. All results were considered statistically significant when p-values were less than 0.05.

Table 1. Baseline Clinicopathologic Characteristics of the 257 Patients Who Underwent Endoscopic Resection of Gastric Neoplasms

Characteristic	Value
Age, yr	61.8±9.4
Sex	
Male	183 (71.2)
Female	74 (28.8)
Family history of gastric cancer*	
No	199 (77.4)
Yes	50 (19.5)
Smoking status	
Never	102 (39.7)
Past	113 (44.0)
Current	42 (16.3)
H. pylori infection status	
Never	30 (11.7)
Past	69 (26.8)
Current	158 (61.5)
Gastric atrophy by serum pepsinogen test*	
Not severe	209 (81.3)
Severe†	44 (17.1)
IM (antrum)	
Absent or mild	125 (48.6)
Moderate or severe	132 (51.4)
IM (body)	
Absent or mild	188 (73.2)
Moderate or severe	69 (26.8)
Type of gastric neoplasm	
Low-grade dysplasia	113 (44.0)
High-grade dysplasia	25 (9.7)
Early gastric cancer	119 (46.3)
No. of neoplasm	
Single	239 (93.0)
Multiple	18 (7.0)
Location of neoplasm	
Upper or middle third	63 (24.5)
Lower third	194 (75.5)
Size of neoplasm, cm	1.2±0.8
Type of endoscopic resection	
Endoscopic mucosal resection	164 (63.8)
Endoscopic submucosal dissection	93 (36.2)

Data are presented as mean±SD or number (%).
H. pylori, *Helicobacter pylori*; IM, intestinal metaplasia.
*The data regarding the family history of gastric cancer were missing in eight patients, and the serum pepsinogen test was not performed in four patients; †Severe atrophy was defined as pepsinogen I ≤30 and pepsinogen I/II ≤2.0.

RESULTS

A total of 257 patients who underwent ER of gastric neoplasm were enrolled. Baseline clinicopathologic characteristics of the patients were shown in Table 1. Low-grade dysplasia, high-grade dysplasia, and EGC were 44.0%, 9.7%, and 46.3%, respectively. The mean follow-up period was 52±29 months and mean 5.0±2.5 times of surveillance endoscopy were performed in each patient during the follow-up period. MGN developed in 19 patients (7.4%). Clinicopathologic characteristics of MGNs were summarized in Table 2. Among 19 MGNs, the rate of gastric cancer was 36.8% (7/19). Except one advanced gastric cancer, all MGNs were treated by ER or operation. However, secondary MGN developed in 16.7% (3/18).

The 5-year cumulative incidence of MGN was 4.8% (Fig. 1). In the univariate analysis, moderate or severe IM in the gastric body was the only risk factor for MGN (hazard ratio, 3.11; 95% confidence interval [CI], 1.24 to 7.80; p=0.016) (Table 3). The 5-year cumulative incidence of MGN in the patients with no/mild corpus IM and in the patients with moderate/severe corpus IM was 1.1% and 10.8%, respectively. The cumulative probability of MGN was significantly different according to the severity of corpus IM (absent or mild vs moderate or severe, p=0.011 by log-rank test) (Fig. 2). In the multivariate Cox proportional hazard model, age, family history of gastric cancer, *H. pylori* infection status, and gastric atrophy by serum PG test were also analyzed with severity of IM, because these variables were suggested as risk factors for MGC in previous studies. The results showed that moderate or severe IM in the gastric body and family history of gastric cancer were independent risk factors for MGN after adjusting for other variables; hazard ratios were

Table 2. Clinicopathologic Characteristics of 19 Metachronous Gastric Neoplasm in Patients Who Underwent Endoscopic Resection of Gastric Neoplasms

Characteristic	No. (%)
Type of MGN	
Low-grade dysplasia	10 (52.6)
High-grade dysplasia	2 (10.5)
Early gastric cancer	6 (31.6)
Advanced gastric cancer	1 (5.3)
Location of MGN	
Upper third	4 (21.1)
Middle third	5 (26.3)
Lower third	10 (52.6)
Treatment modality for MGN	
Endoscopic mucosal resection	13 (68.4)
Endoscopic submucosal dissection	4 (21.1)
Surgery after endoscopic submucosal dissection	1 (5.3)
Chemotherapy	1 (5.3)
Second metachronous neoplasm after treatment of MGN*	
No	15 (83.3)
Yes	3 (16.7)

MGN, metachronous gastric neoplasm.
*One pateint who developed advanced gastric cancer was excluded in this anaylsis.

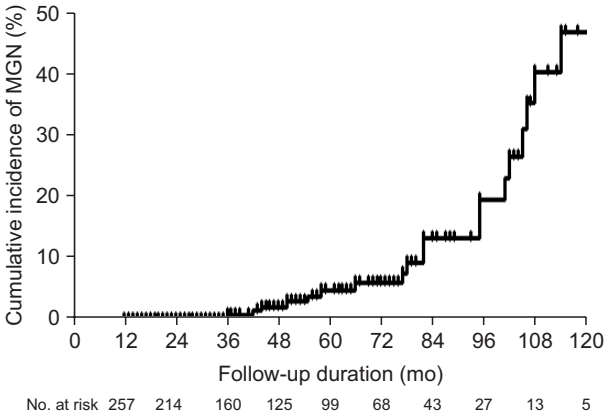

Fig. 1. Kaplan-Meier estimates of the cumulative incidence of metachronous gastric neoplasm (MGN).

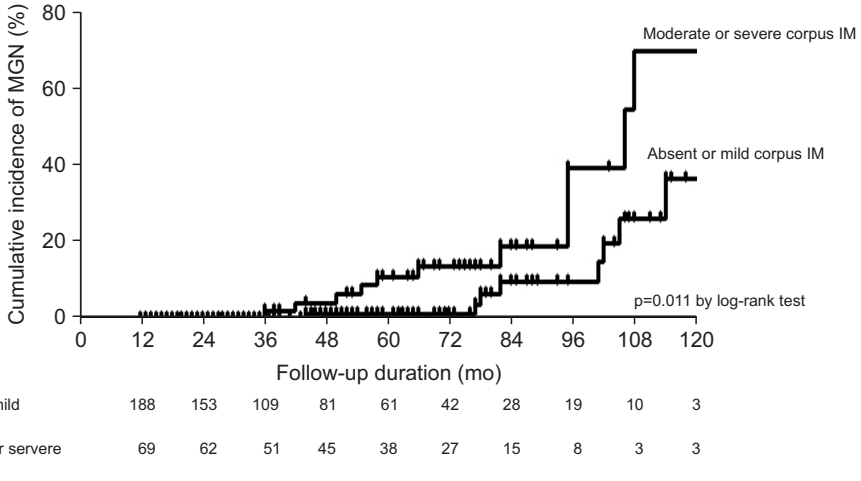

Fig. 2. Kaplan-Meier estimates of the cumulative incidence of metachronous gastric neoplasm (MGN) according to the severity of intestinal metaplasia (IM) in the gastric body.

Table 3. Univariate and Multivariate Cox Regression Analysis of the Risk Factors for Metachronous Gastric Neoplasm

Variable	No.	No. of MGN (%)	Univariate analysis		Multivariate analysis	
			HR (95% CI)	p-value	HR (95% CI)	p-value
Sex						
Female	74	3 (4.1)	1		-	
Male	183	16 (8.7)	1.99 (0.58–6.88)	0.275	-	-
Age, yr						
<65	99	11 (7.9)	1		1	
≥65	158	8 (6.8)	1.29 (0.52–3.22)	0.589	1.22 (0.45–3.31)	0.699
Family history of gastric cancer						
No	199	12 (6.0)	1		1	
Yes	50	6 (12.0)	1.58 (0.59–4.24)	0.364	3.52 (1.09–11.40)	0.036*
Smoking status						
Never	102	7 (6.9)	1		-	
Past	113	7 (6.2)	0.84 (0.29–2.40)	0.745	-	-
Current	42	5 (11.9)	1.90 (0.59–6.14)	0.284	-	-
H. pylori infection status						
Never	30	3 (10.0)	1		1	
Past	69	3 (4.3)	0.51 (0.10–2.54)	0.413	0.63 (0.10–3.86)	0.613
Current	158	13 (8.2)	0.95 (0.27–3.33)	0.930	1.33 (0.27–6.45)	0.724
Gastric atrophy by SPT						
Not severe	209	13 (6.2)	1		1	
Severe†	44	6 (13.6)	2.00 (0.76–5.28)	0.161	1.77 (0.57–5.48)	0.326
IM (antrum)						
Absent or mild	125	8 (6.4)	1		1	
Moderate or severe	132	11 (8.3)	1.74 (0.69–4.34)	0.239	0.84 (0.28–2.54)	0.761
IM (body)						
Absent or mild	188	8 (4.3)	1		1	
Moderate or severe	69	11 (15.9)	3.11 (1.24–7.80)	0.016*	4.12 (1.23–13.87)	0.022*
Type of neoplasm						
Low-grade dysplasia	113	8 (7.1)	1		-	
High-grade dysplasia	25	3 (12.0)	0.84 (0.22–3.26)	0.803	-	
Early gastric cancer	119	8 (6.7)	0.96 (0.25–3.75)	0.951	-	-
No. of neoplasm						
Single	239	17 (7.1)	1		-	
Multiple	18	2 (11.1)	0.894 (0.20–4.00)	0.883	-	-
Location of neoplasm						
Upper or middle third	63	6 (9.5)	1		-	
Lower third	194	13 (6.7)	0.60 (0.23–1.58)	0.299	-	-
Size of neoplasm, cm						
≤2	227	16 (7.0)	1		-	
>2	30	3 (10.0)	2.23 (0.63–7.82)	0.212	-	-

MGN, metachronous gastric neoplasm; HR, hazard ratio; CI, confidence interval; *H. pylori*, *Helicobacter pylori*; SPT, serum pepsinogen test; IM, intestinal metaplasia.
*p<0.05; †Severe atrophy was defined as pepsinogen I ≤30 and pepsinogen I/II ≤2.0.

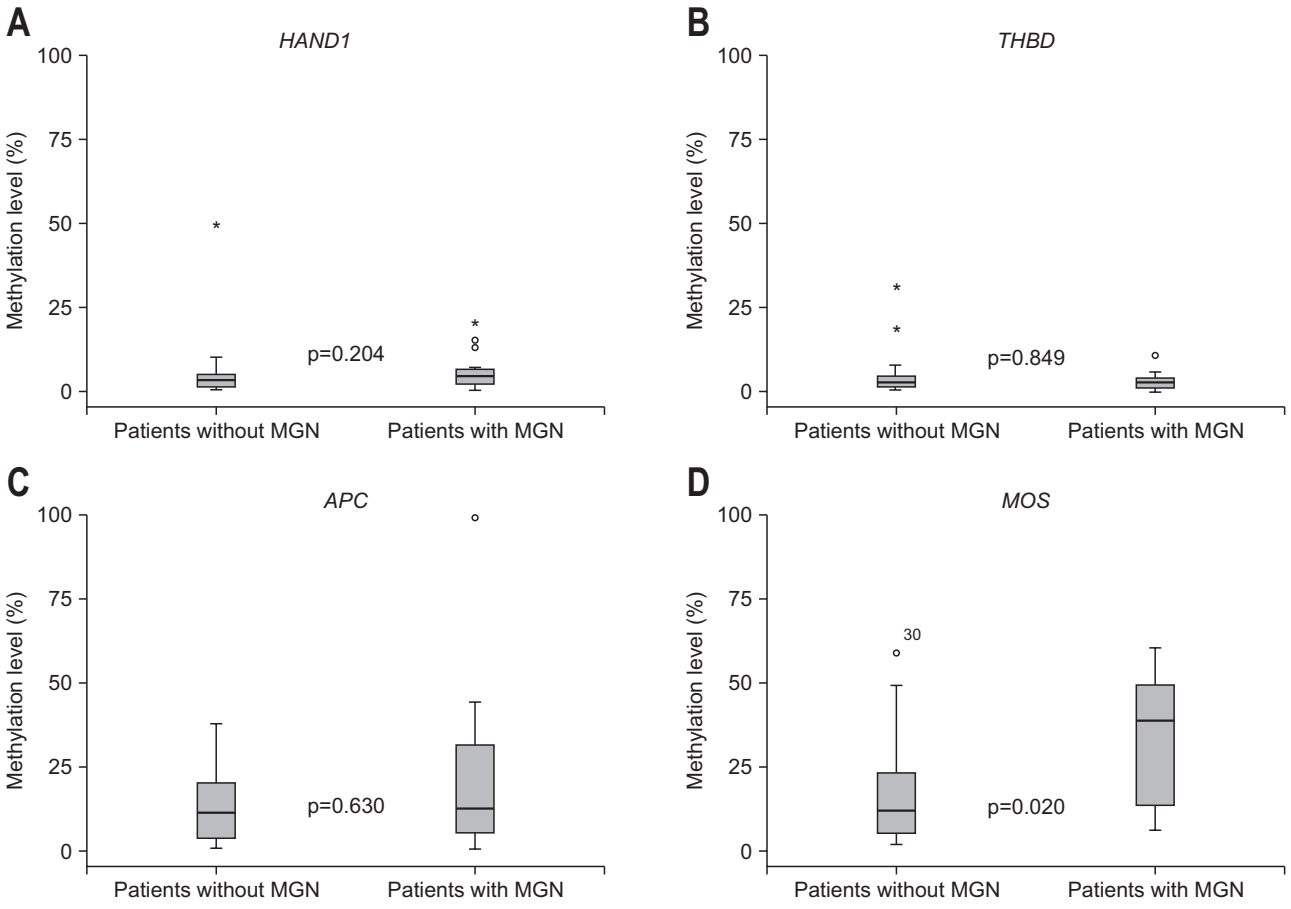

Fig. 3. Methylation levels of the four genes in patients with and without metachronous gastric neoplasm (MGN): (A) *HAND1*, (B) *THBD*, (C) *APC*, and (D) *MOS*.

4.12 (95% CI, 1.23 to 13.87; p=0.022) and 3.52 (95% CI, 1.09 to 11.40; p=0.036), respectively (Table 3).

When the methylation levels of *HAND1*, *THBD*, *APC*, and *MOS* were compared between 19 patients with MGN and 19 age- and sex-matched patients without MGN, by methylation specific RT-PCR, only the methylation level of *MOS* was significantly elevated in patients with MGN as compared to those without MGN (p=0.020) (Fig. 3).

DISCUSSION

Even though there have been several studies regarding risk factors for MGC after ER of EGC, the methods to evaluate *H. pylori* infection, gastric atrophy and IM were not meticulous and the suggested risk factors varied. In addition, only few studies have included dysplasia which is usually endoscopically resected after detection and needs regular surveillance similar to EGC. Therefore, we attempted to elucidate risk factors for MGN using well scrutinized methods together with expanding the inclusion criteria to patients with gastric dysplasia. As a result, moderate or severe IM in the gastric body and family history of gastric cancer were independent risk factors for MGN after adjusting for other variables.

IM is an established strongest risk factor for gastric cancer. An epidemiological study suggested that patients with IM have more than a 10-fold increased risk of developing gastric cancer,[21] and our previous findings were similar, i.e., a 10.9-fold risk.[22] In addition, the odds ratio of gastric cancer was found to be 29.3 for patients with severe IM at a 5-year follow-up.[23] IM tends to appear first at the incisura angularis and extends to the neighboring mucosa in both the antrum and body. Therefore, the severity of IM in the gastric body rather than in the antrum reflects the risk of gastric cancer more exactly.[24] The results of our study, that moderate or severe IM not in the antrum but in the corpus was independent risk factor for MGN further corroborate the fact.

Even though many studies have evaluated risk factors for MGC, studies that properly addressed the relationship between IM and MGC are surprisingly scarce. Recently, a large retrospective cohort study suggested that IM was not a risk factor for MGC in patients who underwent ER of gastric neoplasm including low-grade dysplasia.[25] However, the proportion of the patients with IM was unexpectedly high (98.2%), and because 95.0% of IM was severe metaplasia, evaluation according to the

severity of IM was not possible. Another Korean study which evaluated risk factors for synchronous and metachronous gastric neoplasm in patients who underwent endoscopic submucosal dissection of gastric neoplasm suggested that old age (>65 years) was the only independent risk factor of multiple gastric neoplasm.[26] However, in this study, IM was classified as simply positive or negative and the mean follow-up period was too short (16 months). In addition, other studies which suggested old age as an independent risk factor of MGC did not evaluate severity of IM in the background gastric mucosa.[5,27] Therefore, considering that the prevalence of IM increases with age,[28] there is a possibility that age might be a confounding factor and IM was a true risk factor of MGC in these studies.

In contrast with age, family history of gastric cancer was an independent risk factor for MGN after adjusting for other variables in the present study. These results are consistent with previous studies,[5,29] and imply that family history of gastric cancer increases the susceptibility to gastric carcinogenesis and field cancerization irrespective of other factors like *H. pylori* infection and IM.[30,31]

Several earlier studies reported that gastric atrophy is risk factor of MGC after ER of EGC.[3,27] In the present study, we evaluated the severity of gastric atrophy by serum PG test for two reasons: the endoscopic visual evaluation is subjective with high interobserver variability,[32] and histology has a possibility of sampling errors and is sometimes not-available for evaluation of gastric atrophy. However, unlike IM, gastric atrophy was not an independent risk factor of MGN.

Little is known regarding the relationship between aberrant DNA methylation and the risk of MGN. In the present study, we compared the methylation level of several candidate genes in 19 patients who developed MGN with age- and sex- matched patients without MGN. We found that only the methylation level of *MOS* was significantly elevated in patients with MGN as compared to those without MGN. These results are consistent with our previous studies which suggested that *MOS* may be used as a surrogate marker for gastric cancer risk, since the methylation level of *MOS* is related with severity of IM and more importantly, hypermethylation of *MOS* persists after suppression of *H. pylori* infection.[11-13] The mechanism of epigenetic carcinogenesis through aberrant DNA methylation is usually explained by silencing of tumor suppressor gene resulting from inactivation of promoter regions. Therefore, the relationship between hypermethylation of *MOS* which is a proto-oncogene and increased risk of MGN could be considered unreasonable. However, this paradoxical phenomenon observed in our study implies that *MOS* is a passenger gene rather than a driver gene. Because methylation of driver genes which are directly involved in the gastric carcinogenesis occurs only in a very small fraction of cells, the methylation level of drive genes is very low.[6] In contrast, although passenger genes are unlikely to be causally involved in the gastric carcinogenesis, the methylation level of these genes is high and more apt to be clinically measured.[33] Therefore, to evaluate the degree of epigenetic field defects which reflect risk of gastric neoplasm including dysplasia, these passenger genes could be more promising surrogate markers than driver genes. In the present study, we demonstrated that *MOS* could be a candidate molecular marker predicting MGN in the patients who underwent ER of gastric neoplasm. Because our study clearly implies that patients who have IM and show high methylation level of *MOS* have a high risk of MGN, more intensive surveillance should be performed in these patients. In future, it would be expected that if we discover and combine more biomarkers for risk of MGN, we could narrow the group who needs more intensive surveillance after ER of gastric neoplasm. For example, we are considering combining aberrant methylation of *MOS* and the expression of *CDX2*, whose levels correlated with the IM grade in the gastric body[34,35] to predict risk of MGN.

This study has a limitation in that we could not perform methylation-specific PCR in all patients. Therefore, in order to draw definite conclusions, further study including a higher number of subjects might be needed. In addition, because this study was performed in a geographically restrained population, caution should be taken regarding the conclusions extrapolated in terms of impacting global clinical practice.

In conclusion, in the patients who underwent ER of gastric neoplasm, moderate or severe corpus IM and family history of gastric cancer were independent risk factors for MGN and the methylation level of *MOS* was significantly higher in patients who developed MGN than in those who did not. Therefore, more intensive endoscopic surveillance should be performed in individuals with family history of gastric cancer and moderate to severe corpus IM, and additionally show high methylation level of *MOS* even after ER of gastric neoplasm.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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